(54) Title: ANTIBODY CONJUGATES FOR TREATMENT OF NEOPLASTIC DISEASE

(57) Abstract

Immunoconjugates of an antibody to a 240 kD melanoma tumor associated antigen were prepared. Cytotoxic immunoconjugates such as ZME-018 antibody conjugate are useful for treating proliferative cell diseases such as melanoma as well as other tumors which bear the ZME-018 antigen. Detectably labeled compositions for diagnosis of such diseases are also disclosed.

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ANTIBODY CONJUGATES FOR TREATMENT OF NEOPLASTIC DISEASE

Field of the Invention

The present invention relates generally to the field of immunoconjugates and, more particularly, to the use of immunoconjugates in the treatment of cancer. The invention also relates to the treatment of melanoma with conjugates of monoclonal antibodies (MoAbs) and cytotoxic moieties such as gelonin, a ribosomal inhibiting protein, other plant-derived cytotoxic moieties, or cytotoxic or cytostatic biological response modifiers.

Background of the Invention

The necessity for precisely targeting cancer therapy is critical since adequate tumor response is dependent upon delivery and maintenance of intratumor therapeutic concentrations of drugs. Site-directed therapy has become a goal of several investigators utilizing monoclonal antibodies as specific carriers of therapeutic agents.

Cancer is one of the leading causes of mortality and morbidity in the western world. There are many types of cancer, each with its own characteristics. However, cancers share at least one characteristic in common, they involve defects in the cellular growth regulatory process.

Melanoma, the most virulent of skin cancers, is a highly metastatic disease affecting both sexes and is almost uniformly fatal within five years of diagnosis. Surgical removal of localized malignancies has proven effective only when the disease has not spread beyond the primary lesion. Once the disease has spread, the surgical procedures must be supplemented with other more general procedures to eradicate

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the diseased or malignant cells. Most of the commonly
utilized supplementary procedures such as irradiation or
chemotherapy are not localized to the tumor cells and,
although they have a proportionally greater destructive
5   effect on malignant cells, often affect normal cells to some
extent.

Many tumors express antigens or antigenic determinants
which are either expressed very weakly or not expressed at
all by normal cells. Some tumor cells express antigens
10   which are expressed by embryonic cell types but are not
expressed by normal cells of a mature animal. These
abnormally expressed antigens are known as tumor-associated
antigens. These antigens are specific in that while a
particular antigen may be expressed by more than one tumor,
15   it is usually expressed by all or most cells of the
particular tumors which express it. A tumor cell may
express one or more than one tumor-associated antigen.
These tumor-associated antigens may be expressed on the
surface of the cell (cell surface antigen), may be secreted
20   by the tumor cell (secreted antigens) or may remain inside
the cell (intracellular antigen).

The presence of these tumor-associated antigens has
been utilized to detect, diagnose and localize the tumor.
In some cases the presence of the tumor-associated antigens
25   on the tumor cells has allowed the targeting of specific
drugs and other treatment means specifically to the tumor
cells.

Antibodies are proteins normally produced by the immune
system of an animal in response to foreign antigens or
30   antigenic determinants. Antibodies bind to the specific
antigen to which they are directed. For instance,
antibodies to other melanoma antigens have been utilized to
demonstrate specific tumor localization in man after
systemic and intraperitoneal administration.
35   One method of targeting chemotherapeutic agents to
tumor cells and to diminish their effects on normal cells
has been made possible with the development of MoAbs
directed against antigens on the tumor cells which do not

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occur on normal cells. Monoclonal antibodies directed to
specific antigens or antigenic determinants may be prepared
in large quantities.

Antibodies may be labelled in order to allow their use
for localization and treatment of malignant diseases. Such
radiolabelled monoclonal antibodies to tumor cell surface
antigens have been successfully utilized to image tumors in
patients by external scintography (Deland, Semin. Nucl. Med.
19(3): 158-65 (Review) (1989); Juhl, Hepatogastroenterology
36(1): 27-32 (Review), (1989)). Antibodies, coupled to
drugs, may be used as a delivery system by which the drug is
targeted to a specific tumor cell type against which the
antibody is directed because of the antibodies' unique
ability to localize at the tumor site after systemic
administration. Antibodies may also be coupled to toxins
and thus act as a delivery system to target the toxins
directly to specific tumor cells.

The cytotoxic agents frequently utilized for antibody
conjugates primarily fall into three classes of agents:
toxins, radionuclides and chemotherapeutic agents. Antibody
conjugates with each of these types of agents offer
substantial promise as therapeutic agents but present some
unique problems as well (Frankel, et al. Ann. Rev. Med. 37:
125-142 (1986); Reimann et al., J. Clin. Invest. 82(1):
129-138 (1988).). Immunoconjugates containing plant toxins
also offer a unique advantage to other types of antibody
conjugates because:

1.   Doses of immunotoxins required for antitumor
activity are, in general, much lower than that required for
antibody-drug conjugates.

2.   The conjugation of toxins to antibodies does not
appear to affect antibody affinity.

Gelonin is a glycoprotein (M.W. approximately 29-30,000
Kd) purified from the seeds of Gelonium multiforum. Gelonin
belongs to a class of potent ribosomal-inactivating plant
toxins. Other members of this class of
ribosomal-inactivating plant toxins are the chains of abrin,
ricin and modeccin. Gelonin, like abrin and ricin, inhibits

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protein synthesis by damaging the 60S sub-unit of mammalian ribosomes. Although the A chain of ricin (RTA) has been popular for use in immunotoxins, gelonin appears to be more stable to chemical and physical treatment than RTA (Barbieri

5   et al., Cancer Surv. 1: 489-520 (1982)). Furthermore, gelonin itself does not bind to cells and is, therefore, non-toxic (except in high concentrations) and is safe to manipulate in the laboratory. The inactivation of ribosomes is irreversible, does not appear to involve co-factors and

10   occurs with an efficiency which suggests that gelonin acts enzymatically.

Numerous prior workers have suggested or reported linking cytotoxic agents to antibodies to make "immunotoxins." Of particular interest have been

15   immunotoxins of monoclonal antibodies conjugated to the enzymatically active portions (A chains) of toxins of bacterial or plant origin such as ricin or abrin (Nevelle et al., Immunol. Rev., 62: 75-92 (1982); Ross et al., European J. Biochem. 104 (1980); Vitteta et al., Immunol. Rev. 62:

20   158-183 (1982); Ross et al., Cancer Res. 42: (1982) 457-464; Trowbridge and Domingo, Nature (Cond.) 294: 171-173 (1981)).

Gelonin and ricin are among the most active toxins which inhibit protein synthesis on a protein weight basis. Gelonin is 10 to 1000 times more active in inhibiting

25   protein synthesis than ricin A chain. Peptides like ricin and abrin are composed of two chains, an A chain which is the toxic unit and a B chain which acts by binding to cells. Unlike ricin and abrin, gelonin is composed of a single chain, and, because it lacks a B chain for binding to cells,

30   it is itself relatively non-toxic to intact cells (Stirpe, et al. J. Biol. Chem. 255: 6947-6953 (1980)). Mammalian cells apparently lack the ability to bind and/or to internalize the native gelonin molecule. Conjugates of gelonin with a tumor-targeting monoclonal antibody, such as

35   the monoclonal antibody ZME directed to an antigen present on certain tumor cells such as melanoma cells, provide both a specific method for binding the gelonin to the cell and a route for internalization of the gelonin-antibody complex.

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One of the advantages of using the toxin gelonin over using
toxins such as ricin A chain is this reduced toxicity to
normal tissues as compared to the ricin A chain.
Gelonin-coupled with a monoclonal antibody directed to an
5   anti-tumor associated antigen is an active and selective
immunotoxic agent for tumor therapy.

Previous studies have described a number of
antibody-toxin conjugates containing gelonin (Lambert et
al., J. Biol. Chem. 260: 12035-12038 (1985); Thorpe et al.,
10   Eur. J. Biochem 116: 447-454 (1981); Singh et al., J. Biol.
Chem. 264: 3089-95 (1989); Scott et al., J. Natl. Cancer
Inst. 79: 1163-72 (1987); Tedder et al., J. Immunol. 137(4):
1387-91 (1986)). Recently Ozawa, et al. (Int. J. Cancer 43:
152-157) have constructed a gelonin immunotoxin comprised of
15   antibody B467 which binds to the cellular receptor for
epidermal growth factor (EGF). This B467-gelonin conjugate
was highly cytotoxic for EGF receptor expressing cells but
was non-cytotoxic for receptor-deficient cells. Sivam, et
al. (Cancer Research 47: 3169-3173 (1987)) have made a
20   conjugate of the antimelanoma antibody 9.2.2.7 with gelonin
and compared in vitro and in vivo cytotoxic activity with a
9.2.2.7 conjugate of abrin and ricin A chain. These studies
demonstrated that gelonin conjugates show substantial
cytotoxic effects selectively against antigen-positive cells
25   in vitro. In vivo experiments demonstrated that gelonin
conjugates are not toxic up to 2 mg total antibody
dose/mouse and that multiple I.V. administration of gelonin
immunotoxin significantly retarded the growth of an
established subcutaneous human tumor xenograft in nude mice.
30   Compared to conjugates with abrin and ricin, gelonin
conjugates appeared to have similar potency, better
selectively and tumor localization with more significant in
vivo therapeutic properties.

Since the antibody to which the drug, toxin or
35   radioactive label is coupled binds only to tumor cells
expressing a specific antigen, only the tumor cells are
killed. Conversely, with radiation therapy, radiation from
the radiolabelled compounds is not limited solely to the

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tumor cells in which the radiation is taken up. For
example, metabolic or enzymatic degradation of the antibody
may release the radiolabel and allow it to distribute to
other tissues such as kidneys or bone marrow, causing
unacceptable radiation damage to these organs.
Radiolabelled antibodies suffer from problems which limit or
complicate their use as the therapeutic agents.

Summary of the Invention

The present invention provides immunoconjugates of an
antibody (herein designated ZME-018) which recognizes the GP
240 antigen on melanoma cancer cells. One of the antibodies
(225.28S) discussed by Wilson et al. (Wilson, et al., Int.
J. Cancer 28: 293 (1981)) recognizes this melanoma membrane
antigen. This antigen is identified therein by the
designation GP 240. The antibody 225.28S which binds the GP
240 antigen has been designated and is further referred to
herein as ZME-018. In one embodiment, the antibody is
coupled with a toxin selected from the group consisting of
gelonin, ricin A chain and abrin A chain. In another
embodiment the ZME antibody may be coupled with a cytocidal
drug such as adriamycin or a biological response modifier
such as a lymphokine or cytokine. In another embodiment the
antibody may be labeled with a detectable label such as a
radiolabel, a chemiluminescer, a fluorescer, or an enzyme
label. The cytocidal immunoconjugates are useful to treat
and prevent recurrence of tumor-associated GP 240-bearing
tumors by administration of these cytocidal immunoconjugates
to an individual in need of such treatment. The detectably
labeled ZME immunoconjugates are useful for diagnosis and
localization of tumors by techniques known to those in the
art. These labelled immunoconjugates are also useful to
assay for the presence of the GP 240 antigen in biological
specimens and for localizing the tumor site in vivo by means
known to those of skill in the art.

One of the objects of the present invention is to
provide a cytotoxic composition which would specifically
bind to and kill tumor cells. Particularly, it is an object
of the present invention to provide a cytotoxic composition

which would specifically bind to and kill tumor cells which express the GP 240 antigen as described above. Antibody ZME-018 was prepared at Hybritech, Inc. using salt fractionation and DEAE chromatography and was judged homogenous by SDS PAGE (Wilson et al., *Int. J. Cancer 28*: 293-300 (1981)). Another aspect of the invention concerns a method of killing human melanoma cells, or any other tumor cells expressing the ZME (GP 240) antigen, by contacting the cells with a cytocidally effective amount of an immunotoxin.

It is a further object of the present invention to provide such a composition which would be toxic to tumor cells but cause minimal injury to normal tissue.

Description of the Drawings

Figure 1 demonstrates the coupling and purification schema for ZME-gelonin.

Figure 2 demonstrates the purification of ZME- gelonin by S-300 gel permeation chromatography.

Figure 3 demonstrates the elution profile of the Cibachron-Blue sepharose column after the high-molecular weight material from S-300 chromatography was applied and eluted with a linear salt gradient (0-300 mM Nacl). Two protein peaks were demonstrated: a flow-through peak (fractions 14-20) and a bound peak eluted with high salt (fractions 44-75).

Figure 4 demonstrates the electrophoretic pattern of gelonin and ZME gelonin conjugate.

Figure 5 demonstrates comparative ELISA Assay data of ZME (open circles) and ZME gelonin (closed circles).

Figure 6 demonstrates the cytotoxicity of ZME- gelonin and free gelonin on log-phase AAB-527 cells after 72 hour exposure.

Figure 7 demonstrates the cytotoxicity of ZME- gelonin and free gelonin on log-phase AAB-527 cells.

Figure 8 demonstrates the cytotoxicity of ZME- gelonin on antigen positive target melanoma cells (AAB-527) and antigen negative T-24 cells in culture.

Figure 9 demonstrates the influence of free antibody on ZME-gelonin cytotoxicity.

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Figure 10 demonstrates the effect of IFN-α, IFN-γ and TNF on ZME-gelonin cytotoxicity. Closed circles show the dose-response for ZME-gelonin alone. Open diamonds show the dose-response for ZME-gelonin plus IFN-γ. Open triangles

5   show the dose-response for ZME-gelonin in the presence of a fixed amount of TNF-α. Closed circles with dotted lines show ZME-gelonin dose-response curve in the presence of a fixed amount of IFN-α.

Figure 11 demonstrates the effect of ZME-gelonin on

10   antigen positive (A-375, closed circles) and antigen negative (CEM, open squares) cells in a human tumor stem cell assay.

Figure 12 demonstrates the cytotoxic effect of ZME-gelonin on stem cell survival of different lines

15   obtained from fresh biopsy specimens of 4 different patients.

Figure 13 demonstrates the tissue distribution of ZME antibody and ZME-gelonin conjugate in nude·mice bearing human melanoma zenografts.

20   Detailed Description of the Invention

As used herein the term "monoclonal antibody" means an antibody composition having a homogeneous antibody population. It is not intended to be limited as regards the source of the antibody or the manner in which it is made.

25   Melanoma cells express a 240 kD (GP 240) antigen on their cell surface. Antibodies to this antigen have been produced. Antibody ZME-018 (from Hybritech, Inc.) is a murine monoclonal antibody IgG_{2a} recognizing a 240 Kd glycoprotein present on most human melanoma cells.

30   Monoclonal antibodies of the IgG_1, IgG_{2a} and IgG_{2b} isotypes which recognize an epitope of this 240 kD antigen may be produced. This 240 Kd epitope of the ZME antigen will for the purpose of this invention will be designated the ZME epitope. Thus, all antibodies which recognize this ZME

35   epitope are functionally equivalent.

These representative hybridoma cultures whose cells secrete antibody of the same idiotype, i.e., all recognize the ZME epitope, have been deposited at the American Type

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Culture Collection of 12301 Parklawn Drive, Rockville,
Maryland 20852 ("ATCC") and have been assigned the accession
number _____.

These monoclonal antibodies may be made by methods
known to those of skill in the art. The characterization of
and procedure for making the hybridoma cell cultures which
produce these antibodies is described in detail. (Wilson et
al. Int. J. Cancer 28:293 (1981); Imai et al, Transplant
Proc. 12:380-383 (1980)). Briefly, hybridomas were
constructed with the murine myeloma cell line Sp2/0-Ag-14
and splenocytes from mice immunized with the melanoma cell
line M21 as described by Imai et al., ((1980) Transplant
Proc. 12:380-383). The hybridomas secreting the monoclonal
antibody (MoAb) 225.28S and 465.12 have been subcloned and
are propagated in vitro and in vivo. Both monoclonal
antibodies are of the IgG_{2a} subclass and were purified from
mouse ascites fluid by absorption/elution from protein A
Sepharose 4B (Pharmacia, Piscataway, NJ, USA) prior to their
use.

Hybridomas producing antibodies which reacted with
human melanoma cells but not with normal human cells were
further characterized. The antibodies produced by the ZME
cell line and hybridoma producing functionally equivalent
antibodies reacted with the ZME antigen on human melanoma
cells. They also reacted with 70 - 80% of randomly-obtained
melanomas tested, and exhibited no reaction to various
tissues as summarized on Table 1 in Example 4.

As used herein with respect to the exemplified murine
monoclonal anti-human melanoma antibodies, the term
"functional equivalent" means a monoclonal antibody that:
(1) crossblocks an exemplified monoclonal antibody; (b)
binds selectively to cells expressing the ZME antigen such
as human melanoma cells; (c) has a G or M isotype; (d) binds
to the ZME antigen as determined by immunoprecipi- tation or
sandwich immunoassay; and (e) when conjugated to gelonin,
exhibits a tissue culture inhibitory dose (TCID) of at least
50% against at least one of the AAB-527, or A375 cell lines
when used at a dose of 80-100 units per ml.

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Antibody ZME was conjugated to gelonin using
N- succinimidyl-3-(2-pyridyldithio)propionate (SPDP) or
2-iminothiolane (IT) as a coupling agent. The conjugates
were tested against AAB-527 and A375 cells in a 72-hour
tissue culture assay. The antibody conjugates exhibited
acceptable antiproliferative activity (TCID 50% of less than
100 units ml) against both of these cell lines.

Further details of the characterization of the
antibodies are provided in the examples below.

Immunochemicals

The immunochemical derivatives of this invention that
are of prime importance are immunotoxins (conjugates of the
ZME antibody and a cytotoxic moiety or a biological response
modifier) and labelled (e.g., radiolabelled,
enzyme-labelled, or fluorochrome-labelled) derivatives in
which the label provides a means for identifying immune
complexes that include the labeled antibody.

The cytotoxic moiety of the immunotoxin may be a
cytotoxic drug or an enzymatically active toxin of bacterial
or plant origin (gelonin), or an enzymatically active
fragment ("A chain") of such a toxin. Enzymatically active
toxins and fragments thereof are preferred and are
exemplified by gelonin, diphtheria A chain, nonbinding
active fragments of diphtheria toxin, exotoxin A chain (from
Pseudomonas aeruginosa), ricin A chain, abrin A chain,
modeccin A chain, alpha-sarcin, Aleurites fordii proteins,
dianthin proteins, Phytolacca americana proteins (PAPI,
PAPII, and PAP-S), momordica charantia inhibitor, curcin,
crotin, saponaria officinalis innibitor, mitogellin,
restrictocin, phenomycin, and enomycin. Most preferred is
the conjugation with gelonin.

Biological response modifiers which may be coupled to
the ZME antibody and used in the present invention include,
but are not limited to, lymphokines and cytokines such as
IL-1, IL-2, interferons (α, β, or γ) TNF, LT, TGF-β, and
IL-6. These biological response modifiers have a variety of
effects on tumor cells. Among these effects are increased
tumor cell killing by direct action as well as increased

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tumor cell killing by increased host defense mediated processes. Conjugation of antibody ZME to these biological response modifiers will allow selective localization within tumors and, hence, improved anti-proliferative effects while 5 suppressing non-specific effects leading to toxicity of non-target cells.

Cytotoxic drugs which are useful in the present invention include, but are not limited to, adriamycin (and derivatives thereof), cis-platinum complex (and derivatives 10 thereof), bleomycin and methotrexate (and derivatives thereof). These cytotoxic drugs are sometimes useful for clinical management of recurrent tumors and particularly melanoma, but their use is complicated by severe side effects and damage caused to non-target cells. Antibody ZME 15 may serve as a useful carrier of such drugs providing an efficient means of both delivery to the tumor and enhanced entry into the tumor cells themselves. In addition, specific antibody delivery of cytotoxic drugs to tumors will provide protection of sensitive sites such as the liver, 20 kidney and bone marrow from the deleterious action of the chemotherapeutic agents. Use of drugs conjugated to antibody ZME as a delivery system allows lower dosage of the drug itself, since all drug moieties are conjugated to antibodies which concentrate within the tumor.

25 Conjugates of the monoclonal antibody may be made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate. HCl, active esters such as disuccinimidyl suberate, aldehydes such as 30 glutaraldehyde, bis-azido compounds such as bis(p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate, and bis-active fluorine compounds such as a 35 1,5-difluoro-2,4-dinitrobenzene.

When used to kill human melanoma cells _in vitro_ for therapeutic or for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a

concentration of at least about 10 nM. The formulation and mode of administration for _in vitro_ use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used.

Cytotoxic radiopharmaceuticals for diagnosing and treating tumors carrying the ZME antigen such as melanoma may be made by conjugating high linear energy transfer (LET) emitting isotopes to the antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

The labels that are used in making labeled versions of the antibodies include moieties that may be detected directly, such as fluorochromes and radiolabels as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels are ^{32}P, ^{125}I, ^{3}H, ^{14}C, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferia, 2,3-dihydrophthalzainediones, horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bis-diazotized benzadine and the like may be used to couple the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels.

The antibodies and labeled antibodies may be used in a variety of immunoimaging or immunoassay procedures to detect the presence of tumors expressing the ZME antigen such as melanoma in a patient or monitor the status of such cancer in a patient already diagnosed to have it. When used to monitor the status of a cancer a quantitative immunoassay procedure may be used. Such monitoring assays are carried out periodically and the results compared to determine whether the patient's tumor burden has increased or decreased. Common assay techniques that may be used include direct and indirect assays. Direct assays involve incubating a tissue sample or cells from the patient with a labeled antibody. If the sample ZME antigen bearing cells includes melanoma cells, the labeled antibody will bind to

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those cells. After washing the tissue or cells to remove unbound labeled antibody, the tissue sample is read for the presence of labeled immune complexes.

For diagnostic use the antibodies will typically be distributed in kit form. These kits will typically comprise: the antibody in labeled form in suitable containers, reagents for the incubations and washings, and substrates or derivatizing agents depending on the nature of the label. Antigen ZME controls and instructions may also be included.

Administration of the immunotoxins of the present invention to an individual who has been diagnosed as having a tumor with the ZME antigenic determinant will allow targeting and concentration of the cytotoxic agent at the site where it is needed to kill the tumor cells. By so targeting the cytotoxic agents, non-specific toxicity to other organs, tissues and cells will be eliminated or decreased.

When used in vivo for therapy, the immunotoxins are administered to the patient in therapeutically effective amounts (i.e., amounts that eliminate or reduce the patient's tumor burden). They will normally be administered parenterally, preferably intravenously. The dose and dosage regimen will depend upon the nature of the cancer (primary or metastatic) and its population, the characteristics of the particular immunotoxin, e.g., its therapeutic index, the patient, and the patient's history. The amount of immunotoxin administered will typically be in the range of about 0.1 to about 10 mg/kg of patient weight.

For parenteral administration the immunotoxins will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of

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additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The immunotoxin will typically be formulated in such vehicles at concentrations of about 0.1 mg ml to 10 mg ml.

Gelonin toxin was purified from the seeds of gelonium multiflorum by the method of Stirpe, et al. Briefly, gelonin was extracted from the seeds by homogenization in buffered saline solution (pH 7.4). The supernatant was concentrated after dialysis against 5 mM sodium phosphate (pH 6.5) and the gelonin further purified by ion exchange chromatography as described in Example 1. The purity of the gelonin toxin was assessed by high pressure liquid chromatography (HPLC) and sodium dodecylsulphate-polyacylamide gel electrophoreseis (SDS-Page). Gelonin toxin migrated as a single band with an approximate molecular weight of 29-30,000 daltons.

Gelonin toxin activity was measured as described in Example 2 by protein synthesis inhibition in a cell-free system.

Antibody ZME-018 modified with SPDP as described in Example 5 was conjugated with iminothiolane modified gelonin as described in Examples 3 and 6. The gelonin conjugated antibody was purified as described in Example 7 by column chromatography on a Sephadex G-75 column.

The toxicity of the gelonin-conjuated antibody was determined by protein synthesis inhibition and its antiproliferative activity was determined by in vitro and in vivo tests.

The following examples provide a detailed description of the preparation, characterization, and use of the immunotoxin monoclonal antibodies of this invention. These examples are not intended to limit the invention in any manner.

Example 1
Purification of Gelonin

Seeds of Gelonium multiflorum were shelled and the nuts ground in a homogenizer with eight volumes of 0.14 M NaCl containing 5 mM sodium phosphate (pH 7.4). The homogenate

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was left overnight at 4°C. with continuous stirring, cooled
on ice and centrifuged at 35,000 times g for 20 minutes at
0°C. The supernatant was removed, dialyzed against 5 mM
sodium phosphate (pH 6.5) and concentrated using a pm10
5   filter. The sample was layered on a CM-52 ion-exchange
column (20 x 1.5 cm) equilibrated with 5 mM sodium phosphate
(pH 6.5). Material which bound to the ion exchange resin
was eluted with 400 ml of 0 to 0.3 M linear NaCl gradient at
a rate of 25 ml hour at 4°C. Five ml fractions were
10   collected. The fractions were monitored at 280 nm in a
spectrophotometer. The gelonin eluted in about fractions
55-70 and was the last major elution peak. Fractions 55-70
were pooled, dialyzed against double distilled water and
concentrated by lyophilization. The purity and the
15   molecular weight of each preparation was checked on high
pressure liquid chromotography using a TSK 3000 gel
permeation column with 50 mM sodium phosphate buffer, pH 7.4
and 15% sodium dodecylsulphate-polyacrylamide gel
electrophoresis (SDS-page). Gelonin migrated as a single
20   band with an approximate molecular weight of 29-30,000
daltons.

Example 2

Assay of Gelonin Activity

The gelonin activity was monitored in a cell-free
25   protein synthesis inhibition assay. The cell-free protein
synthesis inhibition assay was performed by sequentially
adding to 50 μl rabbit reticulocyte lysate, thawed
immediately before use, mixing after each addition, the
following components: 0.5 ml of 0.2 M Tris HCl (pH 7.8),
30   8.9 ml of ethylene glycol, and 0.25 ml of 1 M HCl).

Twenty microliters of a salt-amino acid-energy mixture
(SAEM) consisting of: 0.375 M KCl, 10 mM $Mg(CH_3CO_2)_2$, 15 mM
glucose, 0.25-10 mM amino acids (excluding leucine), 5 mM
ATP, 1 mM GTP, 50 mM Tris-HCl (pH 7.6), 10 ul Creatinine
35   phosphate-creatinine phosphokinase, 8 ul ^{14}C leucine
(Amersham, 348 mCi mmol), and adding 1.5 ul of solutions
containing varying concentrations of the gelonin mixture.
The mixture was incubated for 60 minutes at 30°C.

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^{14}C-leucine incorporation was monitored in an aliquot of the mixture by precipitating synthesized protein on glass fiber filters, washing in 10% TCA and acetone, and monitoring the radioactivity in a Beta-counter using Aquasol scintillation fluid. Gelonin with a specific activity no lower than 4×10^9 U/mg was used for conjugation with the antibodies. A unit of gelonin activity is the amount of gelonin protein which causes 50% inhibition of incorporation of [^{14}C] leucine into protein in the cell free assay.

Example 3

Modification of Gelonin With Iminothiolane

Gelonin in phosphate buffered saline was concentrated to approximately 2 milligrams/ml in a Centricon 10 microconcentrator. Triethanolamine hydrochloride (TEA/HCl), pH 8.0 and EDTA were added to a final concentration of 60mM TEA/HCl and 1mM EDTA pH 8.0. 2-Iminothiolane stock solution (20mM) was added to a final concentration of 1 mM and the sample was incubated for 90 minutes at 4°C. under a stream of nitrogen gas.

Excess iminothiolane was removed by gel filtration on a column of Sephadex G-25 (1 x 24cm) pre-equilibrated with 5 mM bis-tris acetate buffer, pH 5.8 containing 50 mM NaCl and 1 mM EDTA. Fractions were analyzed for protein content in microtiter plates using the Bradford dye binding assay. Briefly, forty microliters of sample, 100 ul of phosphate buffered saline (PBS) and 40 ul of dye concentrate were added to each well. Absorbance at 600mm was read on a Dynatech Microelisa Autoreader. Gelonin elutes at the void volume (about fractions 14-20). These fractions are pooled and concentrated by use of a Centricon-10 microconcentrator.

Example 4

Preparation and Characterization of Monoclonal
Antibody to ZME Melanoma Antigen

Antibody-Secreting Hybridomas

One 8-week-old female BALB/c St mouse (SCRF Breeding Colony, La Jolla, Calif.) was injected intraperitoneally (i.p.) with 10^7 human melanoma M21 cells and boosted i.p. with 5×10^6 M21 cells 2 weeks later. One

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50-week-old male NZB/B mouse (SCRF Breeding Colony) was
primed with 5×10^6 BW5 melanoma cells and boosted with 5
injections of 5×10^6 BW5, M51, Colo 38, BW5 and M21
melanoma cells at monthly intervals. Three days after the
5   booster injection, the mouse was sacrificed, and the spleen
was removed, splenocytes were dissociated with a scalpel to
make a cell suspension. The spleen cell suspension was
treated with 0.17 ∂ NH_4Cl in 0.01 M Tris, pH 7.2, for 10 min
to lyse the red blood cells. Then, these splenocytes were
10   fused with SP2/0Ag14 cells as described by Gefter et al.
((1977) Somat. Cell Genet. 3:231.) with the following minor
modifications: 5×10^7 spleen cells and 10^7 SP2/0Ag14 cells
were hybridized with 0.3 ml of 30% (v/v) polyethylene glycol
1000 (PEG) (Baker Chemical Co., Phillipsburg, N.J.) in MEM.
15   After incubation with PEG the cells were washed, and
cultured at a concentration of 2×10^6 cells/ml in D-MEM
overnight. The next day, the cells were suspended in 40 ml
HAT medium and pipetted into about 400 wells (0.1 ml/well)
of microtiter plates (Costar #3596, Cambridge, Mass.) One
20   drop (approximately 25ml) of HAT medium was added at weekly
intervals. After 2-3 weeks, the hybridomas selected for
further studies were cultured in D-MEM with 10% FBS.
Hybridomas were expanded in tissue culture and were grown in
the peritoneal cavity of BALB/c mice primed with 0.5 ml of
25   Pristane (Pfaltz and Bauer, Inc., Stamford, Conn.). The
spent culture medium and ascitic fluid were used as source
of antibody.

Clones of the hybridoma were grown in vitro according
to known tissue culture techniques such as is described by
30   Cotten, et al., Eur. J. Immunol. 3: 136 (1973).

Hybridomas producing antibodies which reacted with
human melanoma cells but not with normal human cells were
further characterized.

As shown on Table 1, they did not react with most
35   normal tissues tested. The antibodies produced by the ZME
cell line and hybridomas-producing functionally equivalent
antibodies reacted with the ZME antigen on human melanoma
cells such as M-21.

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TABLE 1

NORMAL TISSUE REACTIVITY OF ANTIBODY ZME-018 (225.285)*

TISSUE	REACTIVITY**
Bladder	0/3
Brain Cortex	0/2
Cartilage	0/2
Colon	0/2
Nipples	1/2
Duodenum	0/1
Endometrium	0/1
Kidney	0/2
Liver	0/1
Lung	0/4
Lymph Node	0/3
Mammary Gland	0/3
Ovary	0/1
Pancreas	0/1
Peripheral Blood Lymphocytics	0/4
Peripheral Nerve	0/1
Prostate	0/2
Salivary Gland	0/3
Skeletal Muscle	0/1
Skin	0/5
Spleen	0/1
Stomach	0/3
Thyroid	0/2
Tonsil	0/1
Testes	0/5

*From P. Giacomini, et al. Cancer Research 44:1281-1287, 1984

**Number of samples antigen positive/ number samples tested.

Example 5

Modification of Monoclonal Antibody ZME-018 With SPDP

N-succinimidyl 3-(2-pyridyldithio) (propionate)(SPDP) in dimethylformamide was prepared as a stock solution of 3 mg ml in dry dimethylforamide. Since the crystalline SPDP can undergo hydrolysis, the actual concentration of

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chemically reactive crosslinker was determined by
spectrophotometric methods by analyzing the absorbance at
260 nm in a dual-beam spectrophotometer. The concentration
of SPDP stock is calculated from the following equation:

5      $$\frac{\text{Change in absorbance (260nm)}}{0.02 \times 103 \text{ ml mmol}} \times \frac{(3.01)}{0.01} = \text{mmoles/ml/SPDP}$$

One milligram of monoclonal antibody ZME in 1.0 ml of
phosphate buffered saline (PBS) was added to a glass tube.
SPDP stock solution was slowly added at about a 5-fold molar
10   excess to the tube (approximately 10 ul of stock solution),
mixing constantly. The mixture was incubated for 30 minutes
at room temperature, mixing every 5 minutes during the
incubation period.

Excess unreacted SPDP was removed from the sample by
15   gel filtration chromatography on a Sephdex G-25 column
(1 x 24 cm) pre-equilibrated with 100 mM sodium phosphate
buffer pH 7.0 containing 0.5 mM EDTA (Buffer A). Fractions
(0.5 ml) were collected and analyzed for protein content
using the Bradford dye binding assay (Bradford, _Anal._
20   _Biochem._ 72: 248-254 (1976)). Absorbance (600 nm) was
monitored in a 96-well plate using a Bio-TEK Microplate
autoreader. Antibody eluted at the void volume (fractions
14-20) and these fractions were pooled and kept at 4°C. The
protein was concentrated in a Centricon-30 microcentrator.
25   The Centricon retentate was washed with 100 mM sodium
phosphate buffer, pH 7.0 containing EDTA (0.5 mM). The
antibody was concentrated to a final volume of approximately
0.5-0.75 ml.

<u>Example 6</u>

30   <u>Conjugation of SPDP-Modified Monoclonal Antibody</u>
<u>ZME-018 With Iminothiolane-modified Gelonin</u>

One milligram of purified gelonin (2 mg/ml in PBS)
prepared as described in Example 1 was modified with
iminothiolane as described in Example 3. Monoclonal
35   antibody ZME modified as described in Example 4 was mixed
with an equal weight of gelonin modified as in Example 3.
This proportion corresponded to a 5-fold molar excess of
gelonin as compared to antibody. The pH of the mixture was

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adjusted to 7.0 by the addition of 0.05 M TEA/HCl buffer pH
8.0 and the mixture was incubated for 20 hours at 4°C under
nitrogen. Iodoacetamide (0.1 M) was added to a final
concentration of 2 mM to block any remaining free sulfhydryl
groups and incubation was continued for an additional hour
at about 25°C. The reaction mixture was stored at 4°C.
until purification by gel filtration.

Example 7
Purification of Gelonin-Monoclonal Antibody
15A8 Complexes

Non-conjugated gelonin and low molecular weight
products were removed from the reaction mixtures of Example
6 by gel filtration on a Sephadex S-300 column (1.6 x 31 cm)
pre-equilibrated with PBS.

Reaction mixtures from Example 6 were concentrated to
approximately 1 ml with a Centricon 30 microconcentrator
before loading on the Sephadex column. The column was
washed with PBS. One ml fractions were collected and 50 ul
aliquots are analyzed for protein by the Bradford dye
binding assay (Bradford, Anal. Biochem 72: 248 (1976)).

As shown on Figure 2, free- and gelonin-conjugated
antibody eluted in the void volume (about fractions 28-40)
while, unconjugated gelonin elutes at about fractions 45-65.

To remove unconjugated ZME-018, the high molecular peak
(fraction 28-40) from the S-300 column was applied to an
affinity chromatography column of Blue Sepharose CL-6B (1 x
24 cm) pre-equilibrated with 10 mM phosphate buffer (pH 7.2)
containing 0.1 M NaCl. After sample loading, the column was
washed with 30 ml of buffer to completely elute
non-conjugated antibody. The column was eluted with a liner
salt gradient of 0.1 to 2 M NaCl in 10 mM phosphate buffer
pH 7.2. Protein content of the eluted fractions was
determined by the Bradford dye-binding assay.

Figure 2 demonstrates the elution profile of the S- 300
column and demonstrates that gelonin can be separated from
gelonin-antibody conjugate and unconjugated antibody, both
of which coelute in the first peak (about fractions 28-40).
This elution pattern was confirmed by electrophoresis of 50

ul aliquots on 5-20% gradient non-reducing SDS
polyacrylamide gels as shown on Figure 4. The coupling
mixture was loaded on lane 3. Bands for free gelonin (lane
2), free antibody (lane 1) and for one molecule of gelonin

5   coupled per molecule of antibody and two molecules of
gelonin coupled per antibody molecule are shown. The void
volume peak of the S-300 column containing free antibody and
antibody-gelonin conjugate was loaded on lane 4.

Non-conjugated antibody was removed from the gelonin

10   conjugated antibody by affinity chromatography on a column
(1 x 24 cm) of Blue Sepharose CL-6B pre-equilibrated with 10
mM phosphate buffer, pH 7.2 containing 0.1 M NaCl. After
loading the S-300 eluate sample, the column was washed with
30 ml of the same buffer to completely elute non-conjugated

15   antibody.

Gelonin-conjugated antibody bound to the column and was
eluted with a linear salt gradient of 0.1 to 2 M NaCl in 10
mM phosphate buffer, pH 7.2. The antibody- gelonin complex
eluted at approximately 0.7 M NaCl as shown on Figure 3

20   which depicts the elution profile of the Blue Sepharose
column. Protein content of the eluted fractions was
determined by the Bradford dye binding assay. The
protein-containing fractions were pooled and the elution
pattern confirmed by electrophoresis on a 5 to 20% gradient

25   non-reducing polyacrylamide gel. The electrophoretic
pattern of the ZME-gelonin complex is shown on Figure 4.
The flow-through peak (fractions 14-20) contains only free
antibody (Fig. 4, lane 5) while fractions 50-80, eluted with
high salt, contain ZME-gelonin conjugate free of

30   unconjugated gelonin or antibody (Fig. 4, lane 6). The
final product contained ZME antibody coupled to 1, 2 and 3
gelonin molecules. Average gelonin content was 1.5
molecules per antibody molecule.

The rabbit reticulocyte in vitro translation system

35   described in Example 2 was utilized to estimate the gelonin
activity of the essentially pure gelonin-ZME antibody
complex. One unit of activity in this assay was defined as
the amount of protein required to provide 50% inhibition of

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protein synthesis as compared to untreated controls.
Utilizing this assay, the specific activity of both the
native gelonin and the ZME-gelonin conjugate were determined
to be 2 x 10^8 U/mg and 8.2 x 10^5 U/mg respectively. The
essentially pure gelonin-ZME antibody is active in the
reticulocyte lysate assay. A 1:1000 dilution of the
original sample caused approximately a 50% inhibition of
protein synthesis, i.e., a 50% reduction of the
incorporation of ^{14}C-leucine into protein. Thus, the
activity of the original preparation was 1000 U/ml.

EXAMPLE 8
Cell Culture Methods

ZME antigen-negative human bladder carcinoma (T-24)
human cervical carcinoma or ZME antigen-positive human
metastatic melanoma tumor cells A375M or AAB-527 were
maintained in culture using minimal essential medium
supplemented (MEM) with 10% heat-inactivated fetal bovine
serum plus 100 mM non-essential amino-acids, 2mM
L-glutamine, 1mM sodium pyruvate, vitamins and antibiotics.
Cultured cells were routinely screened and found free of
mycoplasma infection.

A.   Cell Proliferation Assay

Cell lines were maintained in culture in complete
medium at 37°C in a 5% CO_2-humidified air incubator. For
assays with combinations of TNF, immunotoxins, IFNα, and
IFNγ, cultures were washed, detached using versene, and
resuspended in complete medium at a density of 25 x 10^3
cells/ml. Two hundred ml aliquots were dispensed into 96-
well microtiter plates and the cells were then allowed to
adhere. This results in a sparsely seeded population of
cells. After 24 hours the media were replaced with media
containing different concentration of either immunotoxins,
toxins, TNF, IFNq, or IFNa. The cells were incubated for 72
hours and analyzed for relative cell proliferation by
crystal violet staining.

B.   Crystal Violet Staining

Cells were washed 3 times with PBS containing calcium
and magnesium fixed and stained with 20% (v/v) methanol

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containing 0.5% (v/v) crystal violet. Bound dye was eluted
with 150 ul of Sorenson's citrate buffer (0.1M sodium
citrate, pH 4.2-50% (v/v ethanol) for 1 hour at room
temperature. The absorbance was measured at 600 nm using a
Bio-Teck microplate reader. Relative cell proliferation
(RCP) was calculated as follows:

$$RCP = \frac{Mean\ Absorbance\ (Drug\ Treated)}{Mean\ Absorbance\ (Non\text{-}drug\ Treated)} \times 100$$

C.   Human Tumor Colony Assay

        Tumor biopsy specimens were obtained from melanoma
patients during clinically indicated biopsy procedures.
Tumor cell suspensions were prepared aseptically (Leibovitz,
et al., Int. J. Cell Cloning 1: 478-485 (1983)).
Additionally, the A375P melanoma and the CEM leukemia cell
lines from the American Type Culture collection (Rockville,
MD) were also tested. Testing for the effects of
ZME-gelonin on the fresh melanoma cell suspensions and cell
lines was assessed in HTCA using standardized procedures for
tumor cell plating in semisolid medium (agarose) in the
presence of complete medium containing 10% fetal calf serum,
each 0.5 ml culture plate containing 100,000 cells for fresh
tumors and 10,000 cells for the cell lines (Hamburger, et
al., Science 197: 461-463 (1977); Salmon, et al., N. Engl.
J. Med. 298: 1321-1327 (1978); Salmon, et al., J. Clin.
Oncol. 7: 1346-1350 (1989)). ZME-gelonin prepared as
described above was tested by addition to the culture plates
shortly after tumor cell plating. ZME-gelonin was added to
triplicate plates at each of four concentrations 0.025 ng ml
to 250 ng ml. In addition to untreated control plates,
unconjugated ZME-18 monoclonal antibody and free gelonin
were tested in parallel. Cell lines and tumor cell cultures
were incubated for an average of 10 days at 37°C in 5% CO_2
in air in a humidified incubator, and colony formation
evaluation with a viability stain (Shoemaker, et al., Cancer
Res. 45: 2145-2153 (1985)) and an automated image analysis
instrument optimized for colony counting (Salmon, et al.,
Int. J. Cell Cloning 2: 142-160 (1984)). Percent survival
of ZME-018 treated cultures in relation to simultaneous

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untreated controls were determined in the same experiments. Dose-response curves were then plotted graphically.

Example 9

Comparison of Binding of Gelonin-conjugated
and Unconjugated ZME Antibody to Target Cells

The ability of the gelonin-conjugated and unconjugated ZME antibody to bind to target cells was assessed. The binding of ZME-gelonin immunotoxin to antigen positive (AAB-527 cells) or antigen negative (T-24 cells) was tested by ELISA assay.

Fifty thousand target cells (AAB-527 cells) or non-target (T-24 cells) were added to each well of microtiter plate. The cells were dried on the plates overnight at 37°C. The cells were then washed with three changes of cold PBS and air dried overnight. The cell surface antigenic determinants remain antigenically active after this treatment.

After attachment of the cells, the plates were washed with Washing Buffer (9.68 g Tris, 64.8 g sodium chloride, 16 ml Tween 20, 800 mg thimerasol in 8 l of double distilled water). Antibody samples were diluted in Washing Buffer containing 1% bovine serum albumin (w/v) (Diluting buffer). Fifty microliters of various concentrations ranging from .02 to 50 ug/ml of either conjugated or unconjugated ZME antibody were added to the wells. After incubation for 1 hour at 4°C, the supernantants are removed and the wells washed twice with Washing Buffer.

Fifty microliters per well of horseradish peroxidase conjugated goat anti-mouse IgG obtained from Bio-Rad and diluted 1:1000 (v/v) (HPGAM) in Diluting buffer was added to each well. The plates were incubated for 1 hour at 4°C and the wells washed twice with Washing Buffer. After incubation of the plates with 50 ul of Substrate Solution (80 mM citrate phosphate (pH 5.0), 1 mM 2,2'AZINO-Bis (3-ETHYL BENZ-THIAZOLINE-6-SULFONIC ACID) (ABTS) DIAMMONIUM SALT (SIGMA CHEMICAL CO) and 4 ul of 30% hydrogen peroxide) in the dark for 30 minutes at room temperature, 25 ul of 4 N

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sulfuric acid was added to each well. The absorbance at 492 nm was determined on an Elisa reader.

As shown in Figure 5, both native ZME and the ZME gelonin conjugate bound well to target cells after 60 minute exposure. Surprisingly, the ZME-gelonin conjugate bound target cells better than did the native antibody. This increase was not due to modification of the antibody by SPDP since SPDP-modified ZME behaved identically to that of native ZME. The increase was also not due to binding of target cells to the gelonin portion of the molecule since pre-treatment of target cells with native gelonin had no effect on either antibody or immunotoxin binding.

Neither ZME nor ZME-gelonin bound to antigen negative T-24 cells as estimated by ELISA assay.

Example 10
Cytotoxicity of Gelonin and Gelonin-ZME Antibody Complex

Cytotoxicity studies of the ZME-gelonin conjugate were performed on antigen-positive cells after continuous (72 hour) exposure to the immunotoxin or native gelonin. As shown in Figure 6, when antigen-positive AAB-527 cells were exposed to approximately 0.1/nM ZME gelonin, 50% cell death was observed. When cells were exposed to native gelonin, a concentration of 100 nM gelonin was required to reduce the cell number to 50% of the untreated control.

Target cells were then treated with various concentrations of ZME-gelonin or gelonin alone on a unit basis as determined in Example 2. As shown in Figure 7, 50% cytotoxicty was obtained using 50 units/ml of ZME-gelonin conjugate while 1×10^7 units/ml of the free gelonin were required to achieve the same effect.

The effect of ZME-gelonin was determined against antigen-negative T-24 cells in log-phase culture. As shown in Figure 8, gelonin alone produced 50% cytotoxicity in AAB-S27 cells at a concentration of 100 ug/ml, similar to that found on AAB-S27 cells. ZME-gelonin produced 50% cytotoxicity in target T-24 cells at a concentration of 10 μg/ml. However, the ZME-gelonin immunotoxin was not

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cytotoxic against non-target T-24 cells even at the highest
concentration tested.

        In order to further demonstrate that the ZME gelonin
cytotoxicity was mediated through the ZME cell surface
antigen, a fixed dose of ZME-gelonin which achieves 80%
cytotoxicity was added to target log phase melanoma cells in
culture in the presence of free ZME antibody or an
irrelevant antibody (15A8, an antibody that does not bind
to melanoma cells). As shown on Figure 9, the presence of
increasing amounts of ZME antibody suppressed the
cytotoxicity of the ZME-gelonin conjugate while the 15A8
antibody had no effect. Thus, the cytotoxicity of the
ZME-gelonin conjugate was directly mediated by the binding
of the ZME antibody to ZME antigen on the target cell.

EXAMPLE 11

Modulation of ZME-gelonin cytotoxicity with

IFN α, IFN γ and TNF.

        To demonstrate the effects of treatment with various
biological response modifiers on immunotoxin cytotoxicity,
log-phase melanoma cells were treated for 24 hours with
fixed doses of IFN α (200 u/ml), IFNγ (20,000 u/ml) or
rTNF-α (20,000 u/ml). These doses were previously
determined to have minimal effect (approximately 20%)
cytotoxic effect against these cells. The cells were then
treated for 72 hours with various doses of ZME-gelonin. As
shown in Figure 10, treatment with rIFNγ resulted in a
2-fold increase in sensitivity to the ZME-gelonin
immunotoxin. However, pre-treatment with both rIFNα and TNF
both resulted in a 2-log increase in sensitivity to the
immunotoxin. Addition of fixed doses of rIFNα, rIFNγ or
rTNF to antigen-positive cells resulted in augmented
cytotoxicity of the ZME-gelonin toxin. Treatment with
rTNF-α caused the greatest increase in immunotoxin
cytotoxicity followed by rIFNα and rIFNγ.

        Substantial augmentation of ZME-gelonin cytotoxic
effect was observed with pre-treatment of rIFNα and rTNF but
not with rIFNγ. While it has been observed that IFNα and
IFN-γ can up-regulate some melanoma surface antigens such as

P-97, there was little effect of the agent on the high
molecular weight antigen (GP 240) recognized by ZME (Murray,
et al., Proc. Am. Assoc. Cancer Res. 27: 313 (1986);
Greiner, et al., Cancer Res. 44: 3208-3214 (1984); Greiner,
et al. Cancer Res. 46: 4984-4990 (1986); Groacomini, et al.,
J. Immunol. 133: 1649-1655 (1984); Imai, et al., J. Immunol.
127: 505-509 (1981); Murray, et al., J. Biol. Res. Mod. 7:
152-161 (1988).). Therefore, the mechanism of TNFα and IFNα
induced augmentation of ZME-gelonin activity is not clear
but could involve changes in the antibody internalization
rate, changes in the cellular processing of the immunotoxin
or a modulation of any one of several interferon-mediated
enzymes.

Since only cells containing the ZME antigen on their
surface were killed by the gelonin ZME immunotoxin, this
immunotoxin is an efficient method to target and kill ZME
tumor associated antigen containing cells while minimizing
or preventing damage or injury to normal non-tumor
associated antigen-bearing cells.

Example 12

Effect of ZME-Gelonin Immunotoxin
in Human Tumor Colony Assay (HTCA)

The activity of ZME-gelonin was also assessed using the
human tumor colony assay against cells obtained from biopsy
of four patients with melanoma.

In vitro cytotoxicity against human cells in culture
was also assessed in the HTCA described in Example 8C.
Various doses of ZME-gelonin immunotoxin were added to an
antigen positive (A-375 melanoma) and antigen negative CEM
cell lines. Survival of colonies was assessed 72 hours
after addition. As shown in Figure 11, doses of immunotoxin
between 0.25 and 2500 ng ml resulted in almost complete
supression of colony survival of the antigen-positive cell
line (closed circle). ZME-018 and free gelonin alone or
combined together were not cytotoxic. There was no effect
against the antigen-negative line (CEM) even at the highest
concentration of immunotoxin tested (open squares).

The effect of ZME-gelonin against 4 different fresh biopsy specimens is shown in Figure 12. Eighty to 90% reduction in survival of melanoma colony forming cells was found in two specimens at the highest immunotoxin dose tested (250 ng ml). One patient showed 50% inhibition of cell growth at this dose, while one patient showed no cytotoxicity of the immuno-conjugate. Only a modest (25%) reduction in colony number was noted with a third specimen. Growth enhancement was noted in the fourth sample at the highest immunotoxin dose. In addition, growth enhancement was observed in one specimen at low doses, while higher doses produced substantial cytotoxicity. As in the cell line experiments, addition of unconjugated ZME-018 and free gelonin were not cytotoxic at the doses tested.

While the HTSCA assay is not infallable, approximately 75% of clinically active antitumor agents are positive in this test system. Agents inactive in the HTSCA have thus far, proven inactive clinically. Therefore, acivity of the ZME-gelonin conjugate in the HTSCA has a 75% probability of demonstrating positive clinical value.

Example 13

Tissue Distribution of ZME Antibody

The tissue distribution of ^{125}I labeled ZME antibody was compared to relevant immunotoxin (ZME-gelonin) and an irrelevant immunotoxin (15A8-gelonin). Each antibody or antibody-conjugate was administered intravenously in the tail vein to 5 nude mice bearing human melanoma xenografts. Each animal received 10mg of total protein labeled with 0.5 μCi of ^{125}I in a total volume of 100μl of phosphate-buffered saline.

As shown in Figure 13, the irrelevant 15A8-gelonin conjugate did not localize specifically in tumor tissues (T/B ration 0.5). In contrast, both the relevant ZME and ZME-gelonin conjugate demonstrated specific localization (T/B ratios of 2.0 and 1.5 respectively). There was no statistically significant difference in the uptake of ^{125}I into tumor after ZME or ZME-gelonin administration. One skilled in the art will readily appreciate that the present

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invention is well adapted to carry out the objects and
obtain the ends and advantages mentioned, as well as those
inherent therein.   The compounds, methods, procedures and
techniques described herein are presently representative of
5   the preferred embodiments, are intended to be exemplary, and
are not intended as limitations on the scope of the present
invention.   Changes therein and other uses will occur to
those skilled in the art which are encompassed within the
spirit of the invention and are defined by the scope of the
10   appended claims.

What is claimed is:

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Claims

1.   A composition of matter comprising a conjugation
of ZME antibody and a moiety selected from the group
consisting of cytotoxic moieties and detectable labels.

5   2.   The composition of claim 1 wherein said moiety is
selected from the group consisting of a toxin, a cytocidal,
a cytostatic drug, a biological response modifier and a
detectable label.

3.   The composition of claim 2, wherein said moiety
10   is gelonin.

4.   A method of treating proliferative cell diseases
comprising administration of a cytocidally effect dose of
the composition of claim 1 to an individual in need of said
treatment.

15   5.   A method of treating melanoma comprising
administration of a gelonin coupled monoclonal antibody
directed to ZME antigen to an individual in need of said
treatment.

6.   A method of preventing recurrence of melanoma
20   tumors comprising administration of gelonin conjugated
monoclonal antibody ZME to an individual diagnosed as having
a tumor bearing ZME tumor associated antigen.

7.   The method of any of claims 4-6, inclusive,
wherein said individual is a human.

25   8.   A method of enhancing the cytotoxic activity of
immunotoxins comprising administration of a biological
response modifier prior to the administration of an
immunotoxin.

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9.   The method of claim 8 wherein said immunotoxin is selected from the group consisting of a gelonin conjugated monoclonal antibody, a ricin conjugated antibody and a TNF conjugated antibody.

5   10.   The method of claim 9 wherein said antibody is selected from the group consisting of an antibody directed against a cell surface antigen of melanoma cells, a cell surface antigen of breast carcinoma cells, and a cell surface antigen of cervical cancer cells.
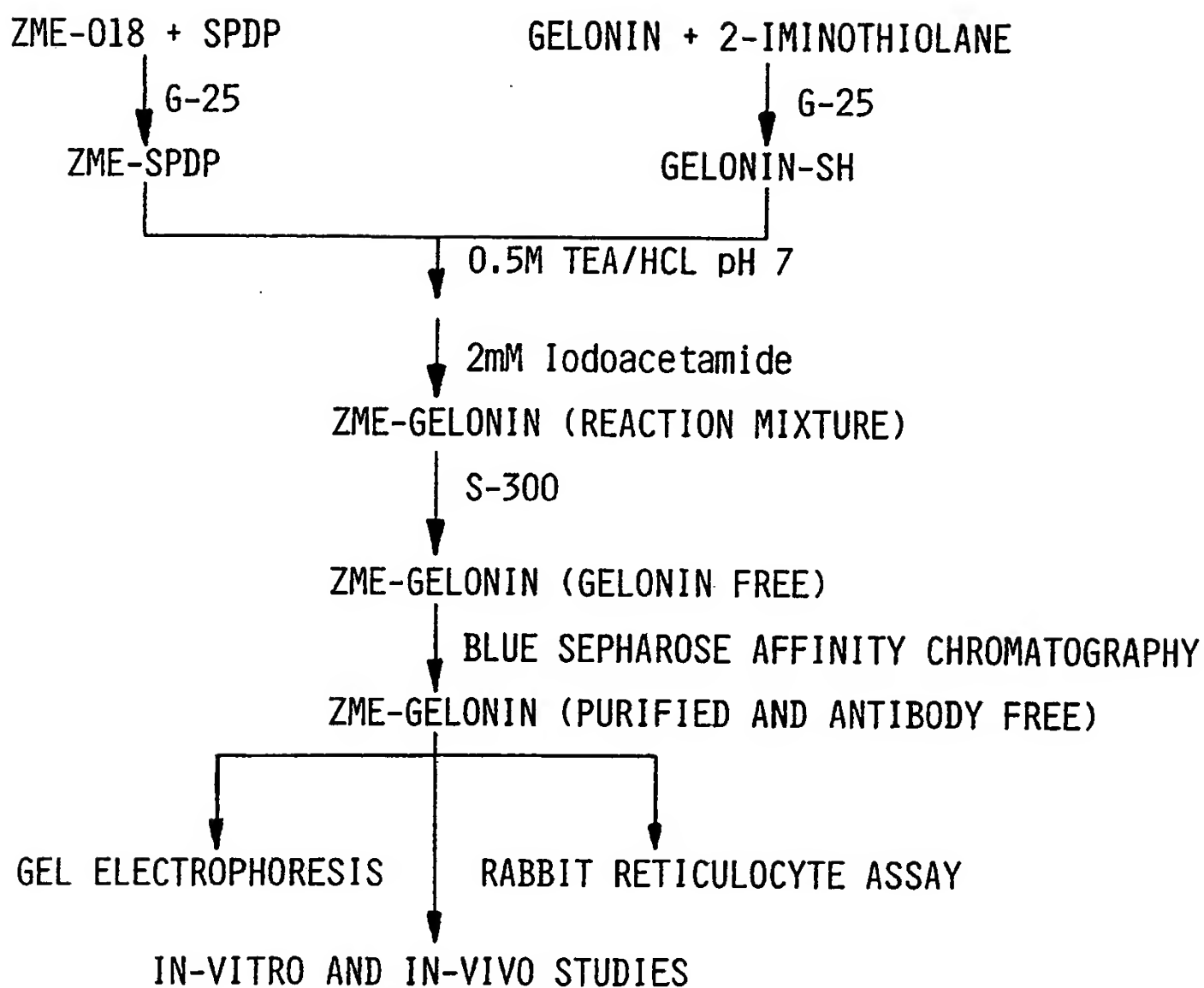
10   11.   The method of claim 10 wherein said antibody is ZME-018.

12.   The method of claim 8 wherein said biological response modifier is selected from the group consisting of IFNa and TNFa.

15   13.   A method of enhancing the cytotoxic activity of immunotoxins comprising administration of a biological response modifier concurrently with the administration of an immunotoxin.
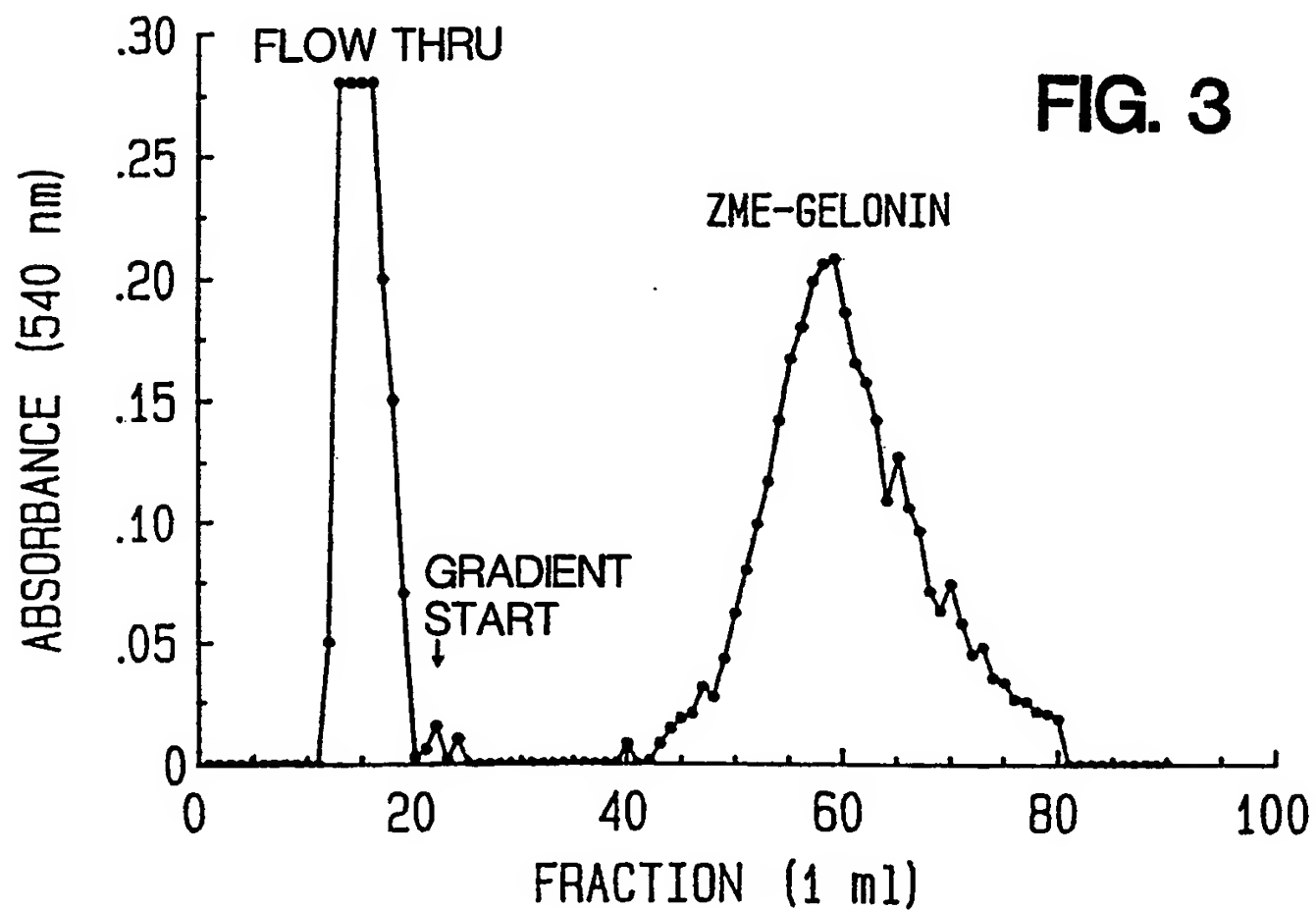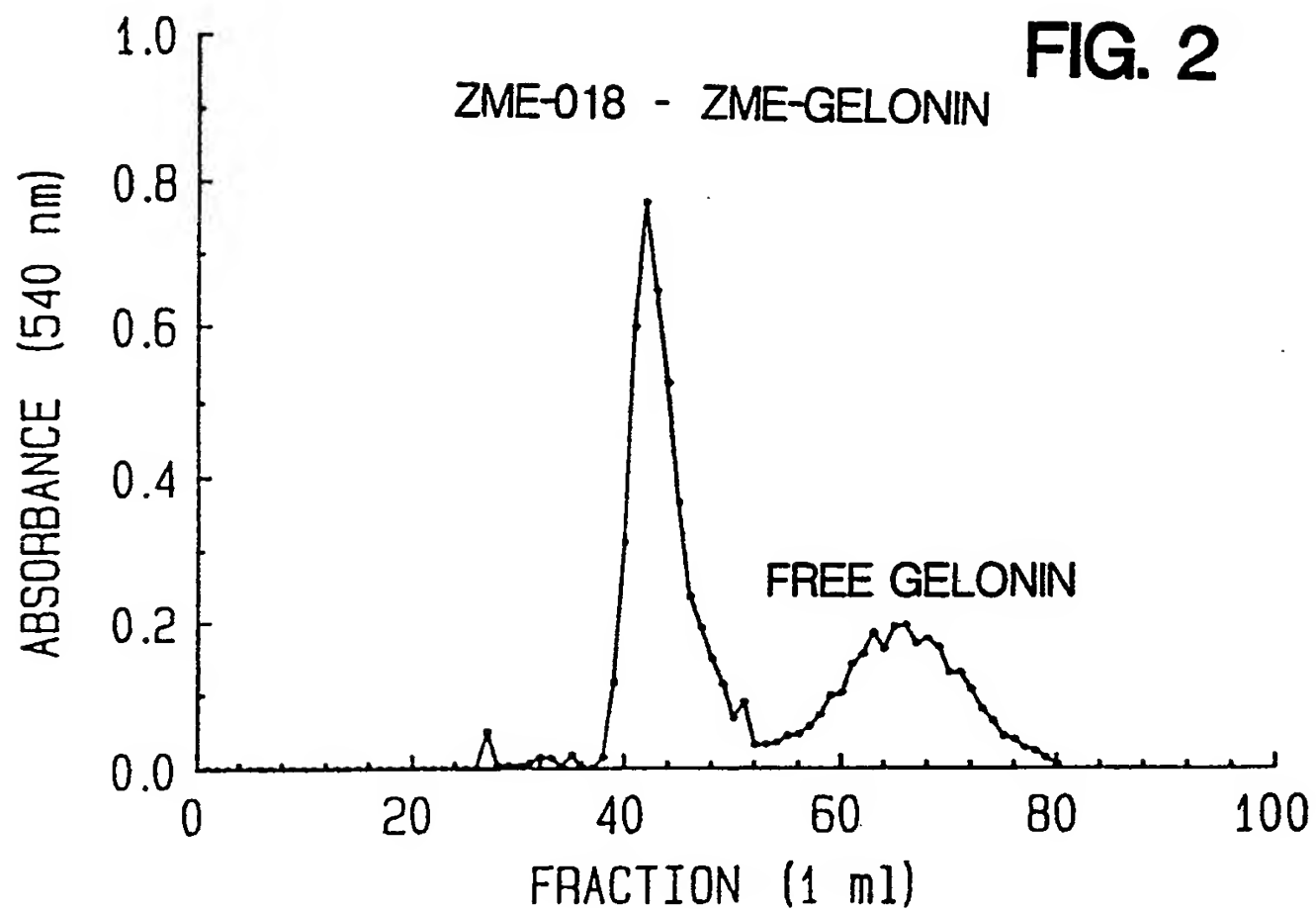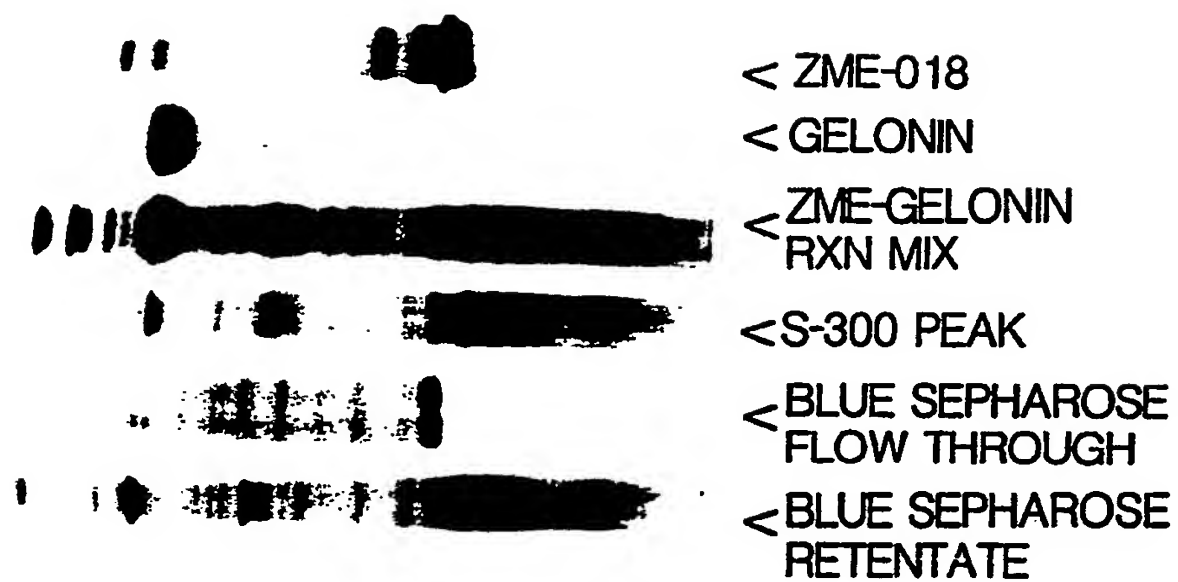
FIG. 1

ZME-018 + SPDP   GELONIN + 2-IMINOTHIOLANE

↓ G-25   ↓ G-25

ZME-SPDP   GELONIN-SH

↓ 0.5M TEA/HCL pH 7

↓ 2mM Iodoacetamide

ZME-GELONIN (REACTION MIXTURE)

↓ S-300

ZME-GELONIN (GELONIN FREE)

↓ BLUE SEPHAROSE AFFINITY CHROMATOGRAPHY

ZME-GELONIN (PURIFIED AND ANTIBODY FREE)

GEL ELECTROPHORESIS   RABBIT RETICULOCYTE ASSAY

IN-VITRO AND IN-VIVO STUDIES

SUBSTITUTE SHEET

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

FIG. 10

FIG. 11

FIG. 12

Y-axis: PERCENT SURVIVAL (1000, 100, 10, 1)

X-axis: CONCENTRATION (ng/ml) (.01, .1, 1, 10, 100, 1000)

FIG. 13

Legend:
☐ ZME-018
■ ZME-GELONIN
▤ 15A8-GELONIN

Y-axis: TISSUE/BLOOD RATIO (0.0, 0.5, 1.0, 1.5, 2.0, 2.5)

X-axis: HEART LUNG LIVER SPLEEN INTESTINE KIDNEY H.QUARTER TUMUR
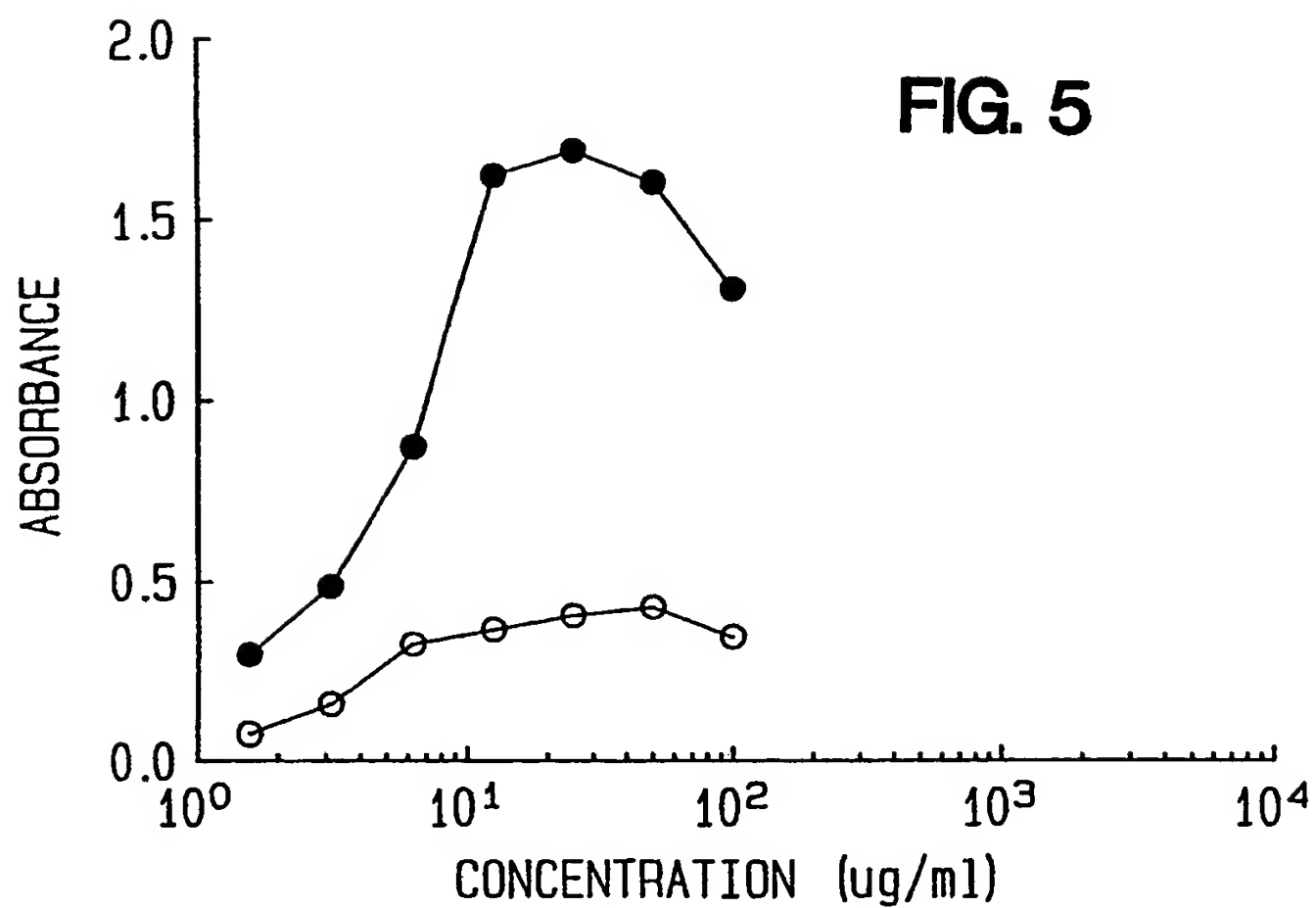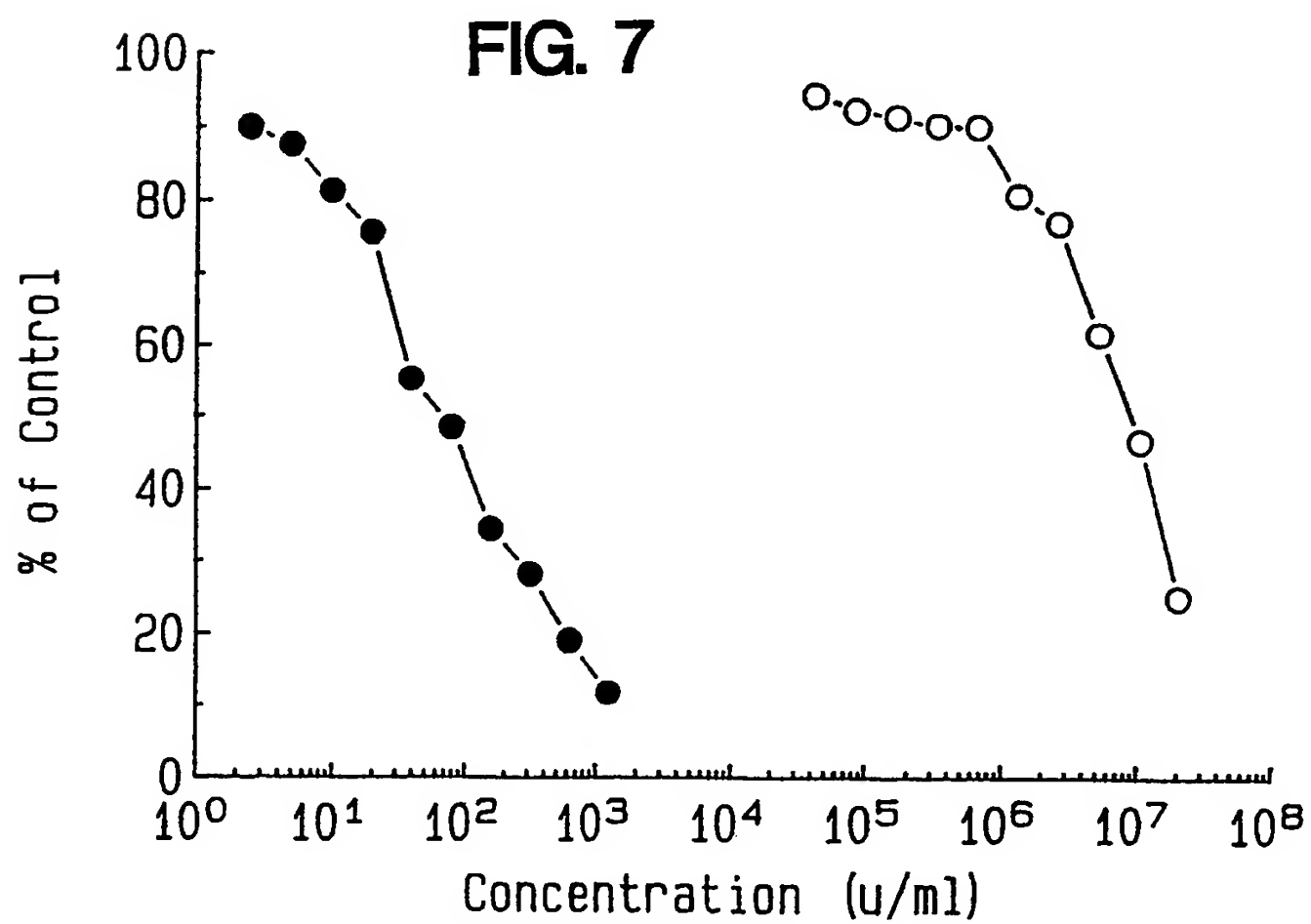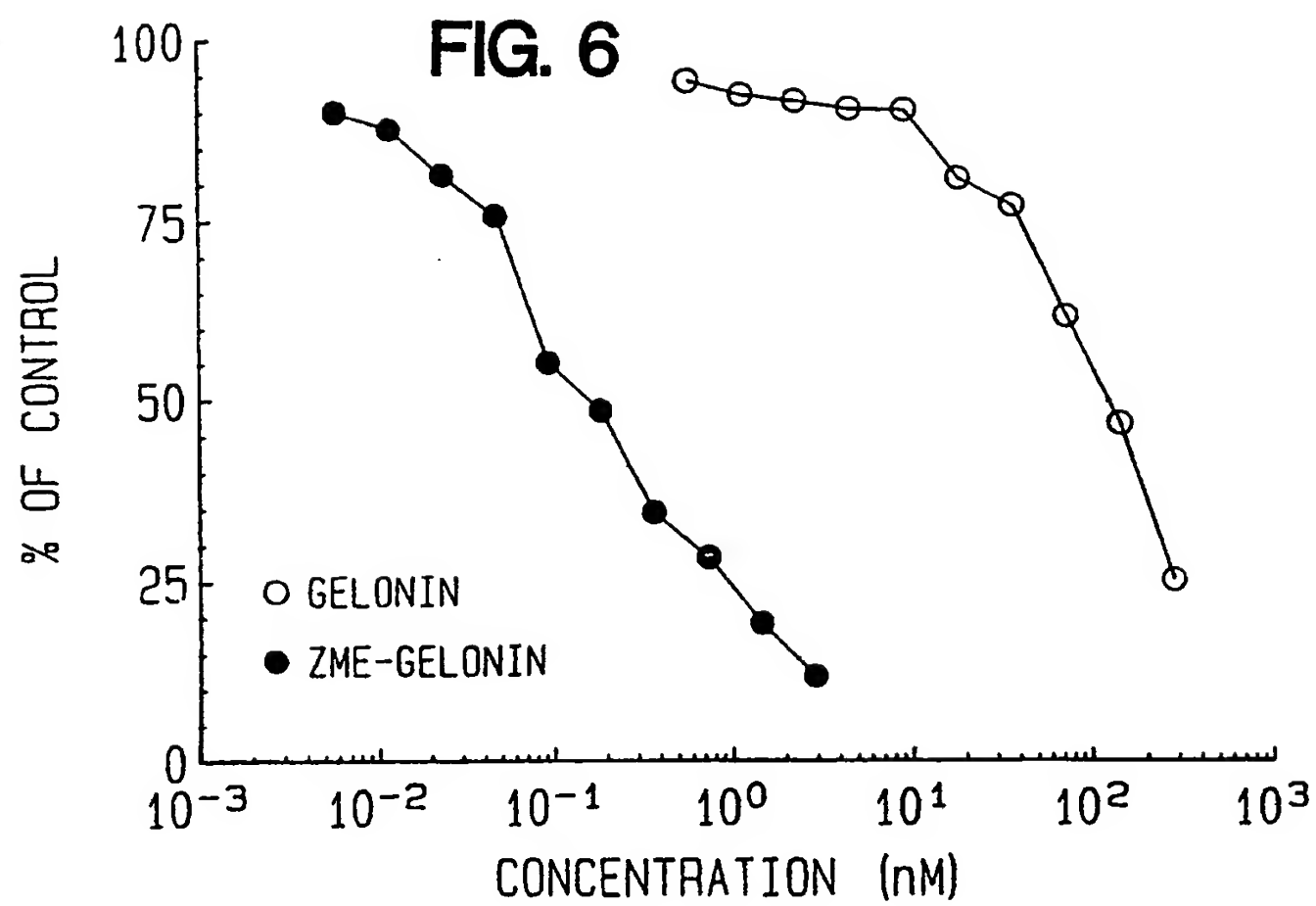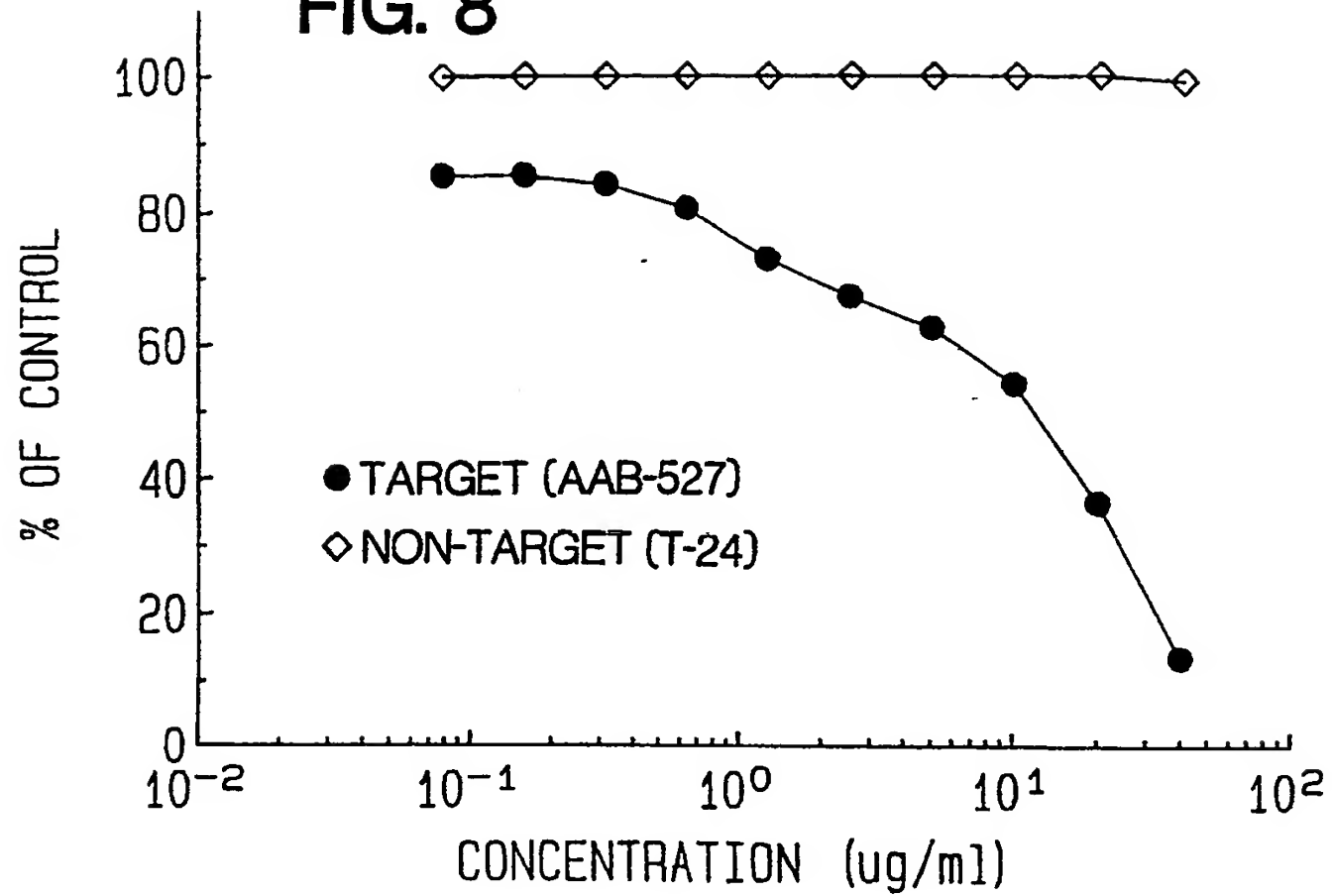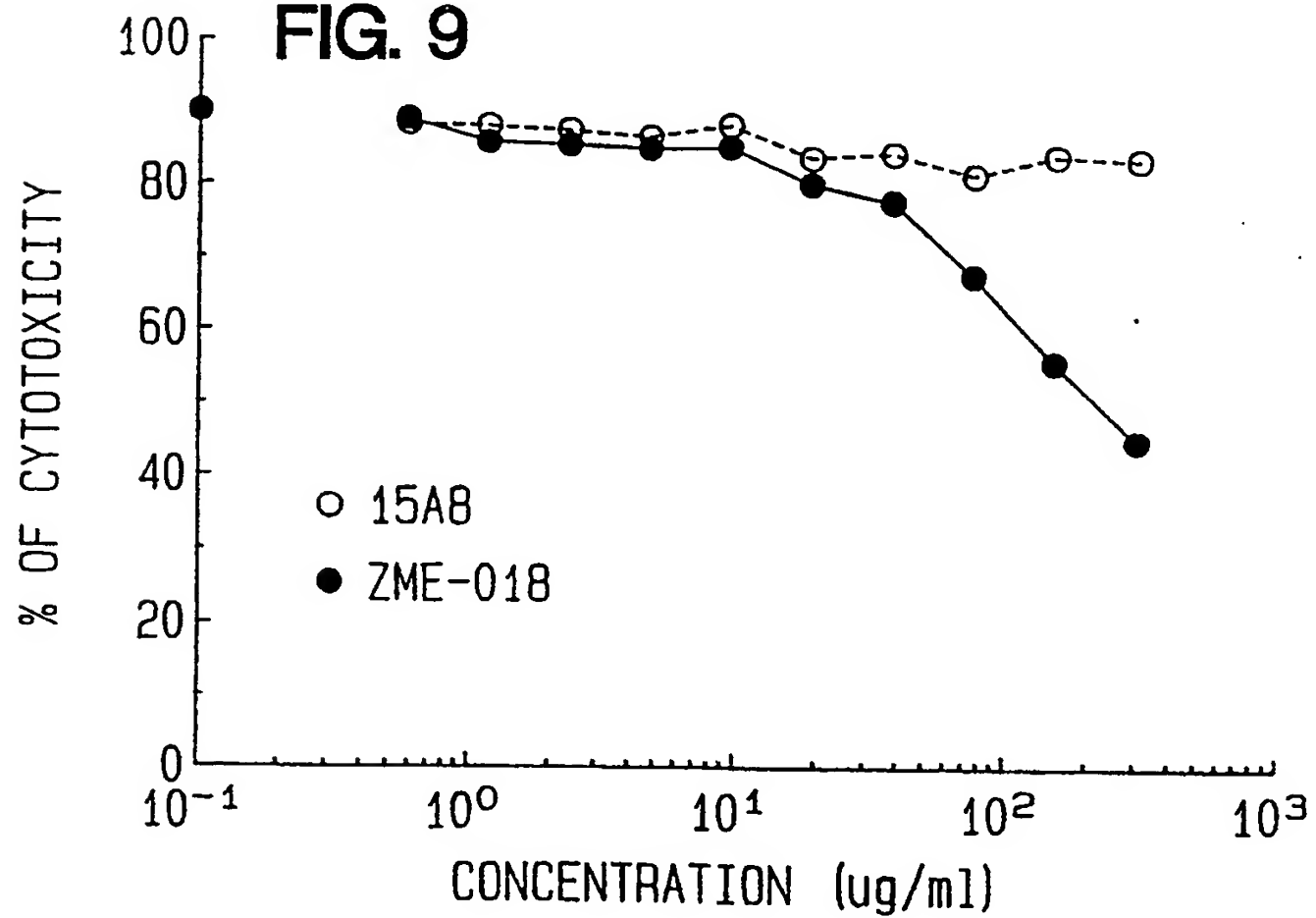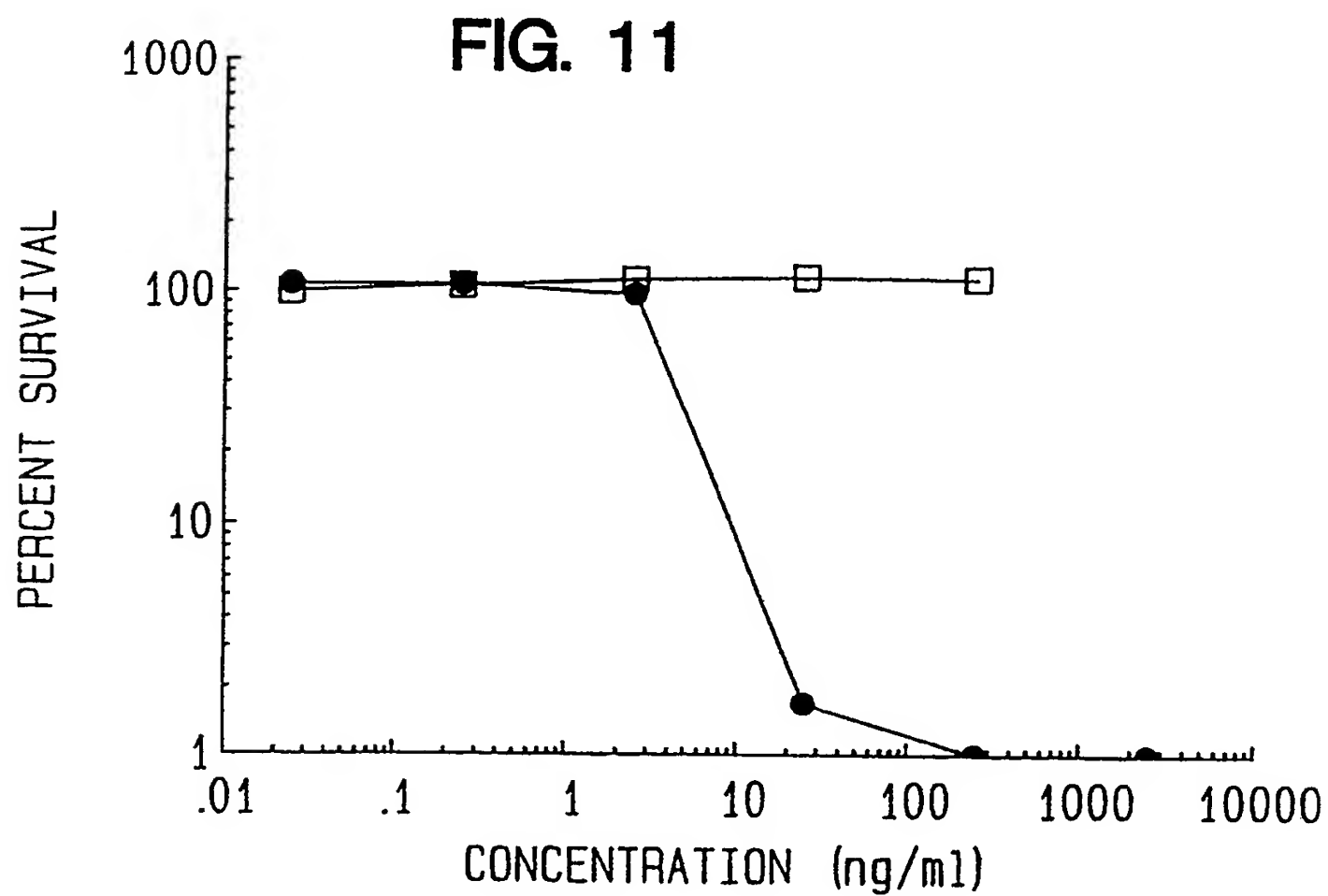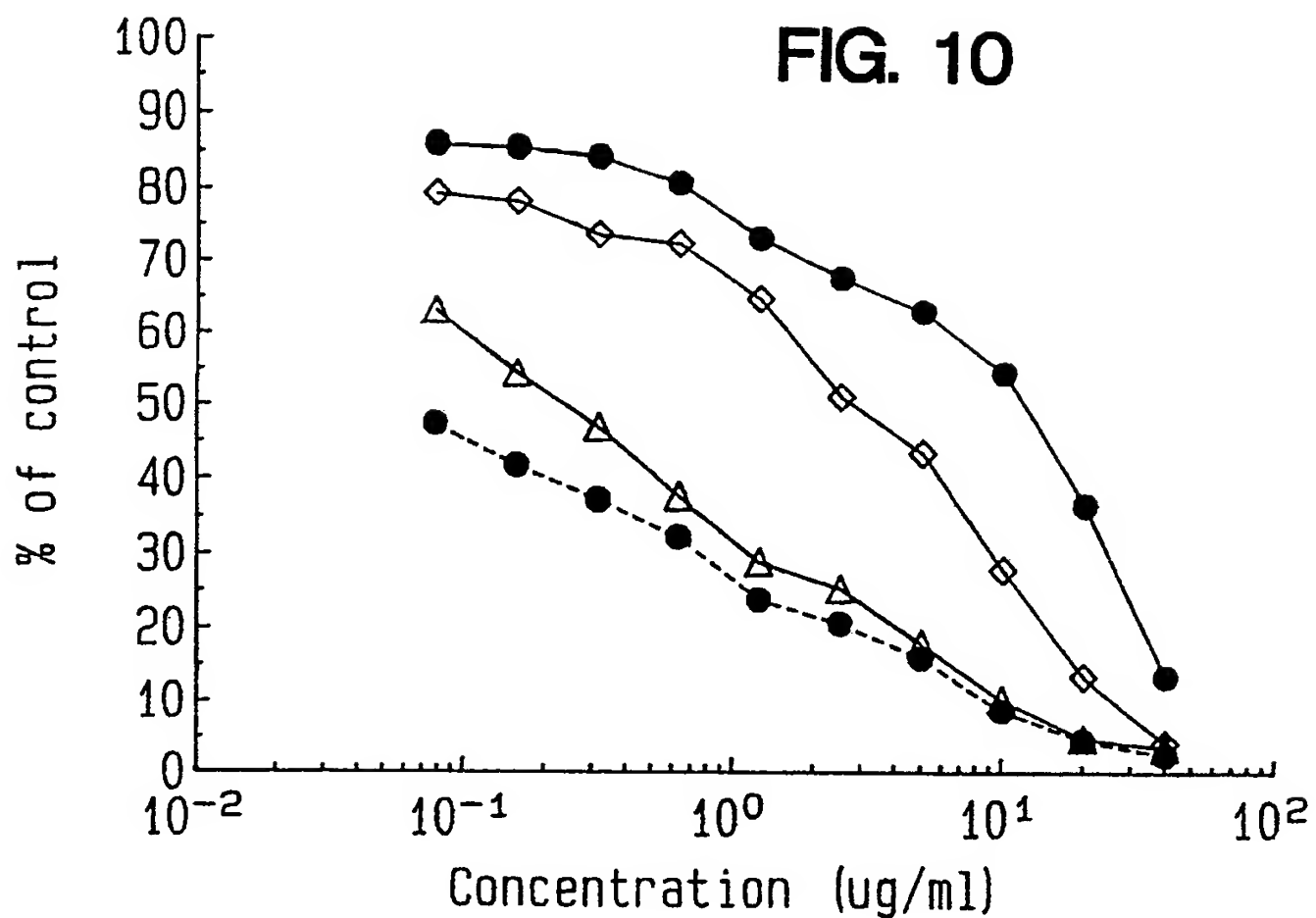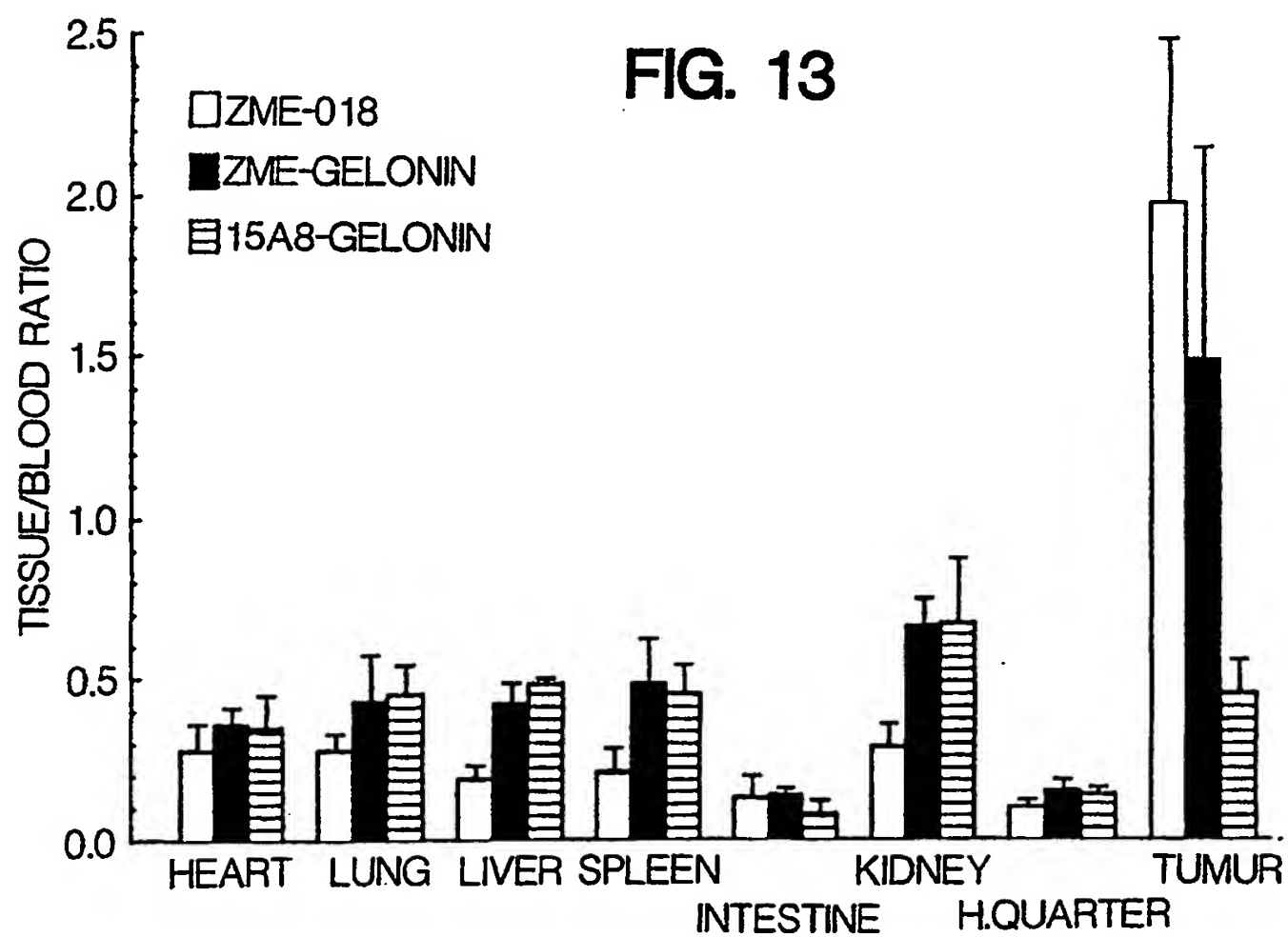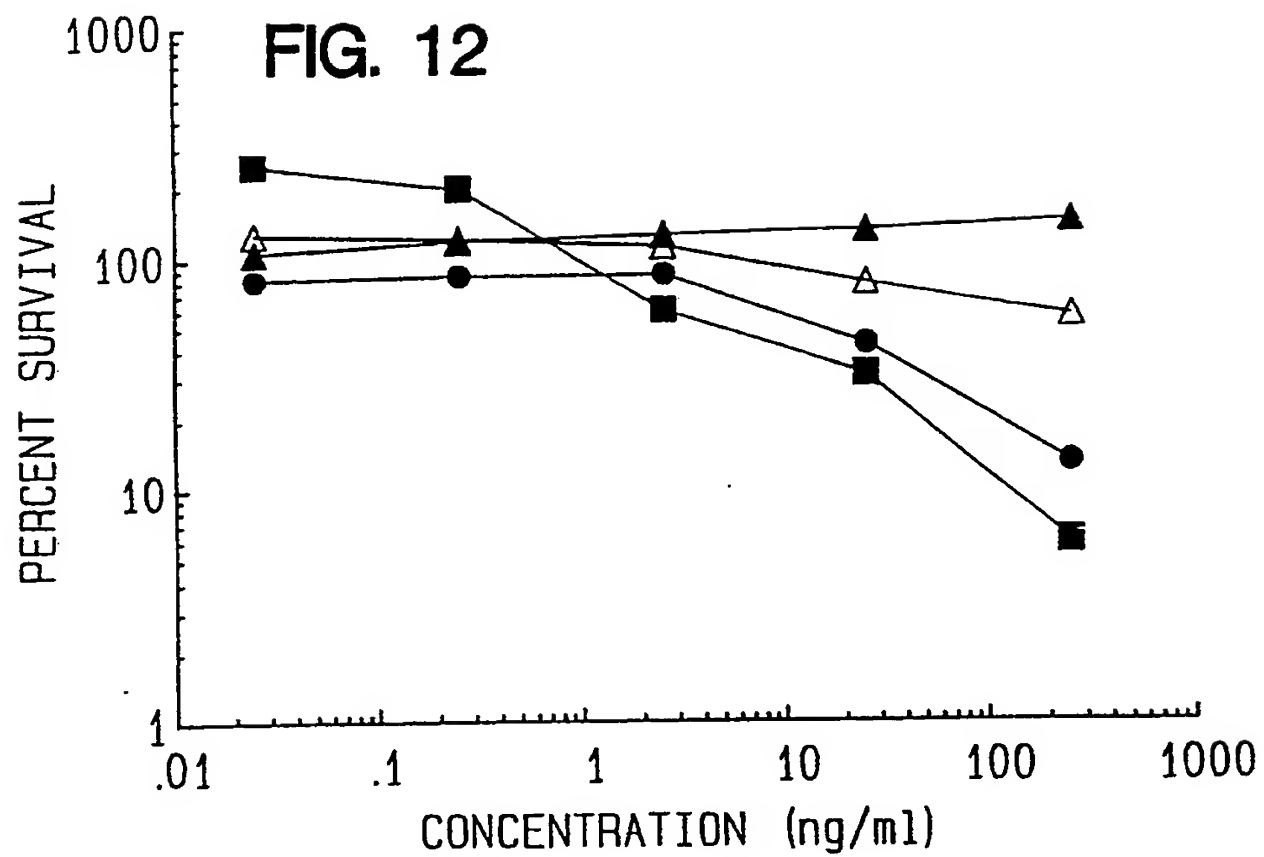
SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application no. PCT/US91/02696

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/66, 37/02, 39/44
USCL.: 424/85.7, 85.1, 85.91

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	424/85.7, 85.1, 85.91, 85.4,; 530/351, 391

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US,A, 4,590,071 (SCANNON ET AL) 20 MAY 1986, SEE COLUMNS 5,6 AND THE ABSTRACT.	1-7
X Y	US,A, 4,863,726 (STEVENS ET AL) 05 SEPTEMBER 1989, SEE COLUMNS 2,5,6.	8-10,13 1-7,11,12
X Y	US,A, 4,894,227 (STEVENS ET AL) 16 JANUARY 1990, SEE COLUMNS 2,4,6.	8-10,13 1-7,11,12
Y	US,A, 4,888,415 (LAMBERT ET AL) 19 DECEMBER 1989, SEE ABSTRACT AND COLUMNS 3,4.	1-7
X Y	US,A, 4,894,225 (ZIMMERMAN) 16 JANUARY 1990, SEE COLUMNS 2-4	8-10,12,13 1-7,11
Y	US,A, 4,894,443 (GREENFIELD ET AL) 16 JANUARY 1990, SEE COLUMN 7 AND CLAIM 3.	1-7
E,Y	US,A, 5,017,371(CUMMINS) 21 MAY 1991. SEE COLUMN 5.	8-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 July 1991	21 AUG 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Kay K. Kim, Ph.D.

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	J. NUCL. MED., VOLUME 28, ISSUED SEPTEMBER 1987, S. MING CHAN ET AL. "COMPARISON OF GALLIUM-67 VERSUS INDIUM-III MONOCLONAL ANTIBODY (96.5, ZME-018) IN DETECTION OF HUMAN MELANOMA IN ATHYMIC MICE", PAGES 1441-1446. SEE PAGE 1441.	1,2 3-13
X Y	BIOLOGICAL ABSTRACTS, VOLUME 84, NO. 10 ISSUED 1987, KIRKWOOD ET AL, "SCINTIGRAPHIC DETECTION OF METASTATIC MELANOMA USING INDIUM-III DTPA CONJUGATED ANTI-GP240 ANTIBODY (ZME-018)" SEE PAGE 628. COLUMN 1, ABSTRACT NO., 99695, J. CLIN. ONCOL. 5 (8): 1247-1255.	1,2 3-13
X,P Y	CANCER RESEARCH, VOLUME 50, NO. 19, ISSUED 01 OCTOBER 1990. PEARSON ET AL, "ENHANCED THERAPEUTIC EFFICACY AGAINST AN OVARIAN TUMOR XENOGRAFT OF IMMUNOTOXINS USED IN CONJUNCTION WITH RECOMBINANT α -INTERFERON", PAGES 6379-6388, SEE PAGE 6379.	13 1-12
X Y	CANCER RESEARCH, VOLUME 50, NO. 1, ISSUED 01 JANUARY 1990, YOKOTA ET AL, "SYNERGISTIC POTENTIATION OF INVIVO ANTITUMOR ACTIVITY OF ANTI-HUMAN T-LEUKEMIA IMMUNOTOXINS BY RECOMBINANT α-INTERFERON AND DAUNORUBICIN", PAGES 32-37, SEE PAGES 32-34.	13 1-12